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STUDIES ON THE PIGMENTATION CHANGES DURING RECONSTITUTION IN TUBULARIA

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A noteworthy feature during reconstitution of the marine hydrozoan *Tubularia* is the appearance of a reddening at the site of hydranth formation. This apparent concentration of pigment suggests a possible relation of the pigment to the metabolic processes of reconstitution.

Moreover, studies on the mechanism of dominance in this form have suggested to Barth (1938) that there is a competition by the ends of a stem piece for something in the circulation. When the circulation is blocked by oil drops (Barth, 1938), glass rods (Rose and Rose, 1941), or ligatures (Barth, 1938), dominance is obliterated. When an open capillary tube of short length is inserted into the coelenteron and the stem ligated about it, thus breaking the coenosarcal connection but temporarily keeping some fluid exchange, a dominance effect was noted by Rose and Rose (1941).

Since pigment granules are very evident in the circulation during regeneration, an investigation of the origin, fate and nature of the pigment was undertaken.

MATERIAL AND METHODS

The work reported here was done on *Tubularia* supplied by the Marine Biological Laboratory at Woods Hole during August, 1951. The stems were four to eight inches long and unbranched. The hydranths show the tentacle characteristics reported for *Tubularia crocea*. The coelenterons were usually partitioned, starting just below the hydranth constriction, by tissue sheets connecting the gastric ridges. These sheets may connect adjacent ridges, in which case the cross-section shows cords less than a diameter, or several sheets may seem to originate from a center giving a wheel-hub effect in cross-section (Fig. 1, C and D). The flagellated cells responsible for fluid circulation are mainly at the two junctions of any gastric ridge with the rest of the coenosarc as seen in cross-section.

The pigment of *Tubularia* is typically red. It has been long observed that this redness is variable but an unusual event, noticed by the Woods Hole Supply Department and by us for the first time in August, 1951, is that interspersed with the red stems were yellow and orange stems, as well as intergrades. These stems were in no apparent way otherwise different from the red ones. The colors occurred in both young and old hydranths, both sexes, and in stems of differing lengths. It

was not unusual to find a yellow stem in the midst of red stems. These individuals offered a new approach to the problem of pigmentation during regeneration.

Unless otherwise noted, all stem fragments were 5 mm. in length and were taken from a point 3 mm. behind the hydranth constriction. To keep track of the original polarity, the distal cut is made horizontal and the proximal oblique. Where a group of four or less stems was being followed, the stems were placed in 50-ml. Erlenmeyer flasks half filled with sand-filtered sea water. Where 5 to 10 stems were

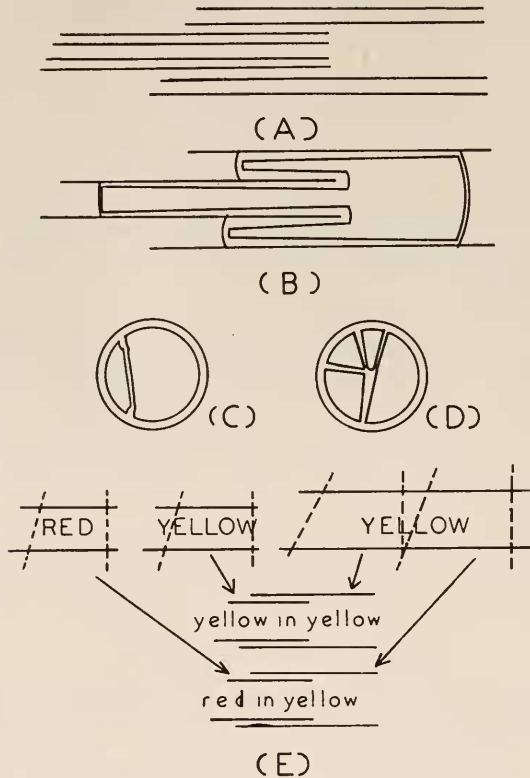


FIGURE 1. For explanation, see text.

being followed as a group, they were kept in fingerbowls in 100 ml. of filtered sea water. All containers were placed in an illuminated cold room at 16–18° C. The average time for regeneration of a hydranth was about three days.

EXPERIMENTAL

Preliminary experiments involving regeneration of sections from hydranths of colors other than red showed that without exception the regenerant was of the same color as the original. In some cases a decrease in color intensity seems to have occurred, but this is not clear cut. An experiment was then performed

wherein portions of red stems were allowed to fuse with yellow stem fragments, to determine the influence of this situation on the color of the hydranth forming in the yellow component.

All yellow stems were taken from animals bearing yellow hydranths, and all red stems from animals bearing red hydranths. Hydranth and stem color always corresponded, although the intensity was both less and somewhat more variable in the stem portion.

For the fusion experiment, stems were selected which were either intensely red or pale yellow. For each set in this experiment, one yellow and one red stem of approximately equal diameter, and a second yellow stem of larger diameter were used (Fig. 1, E). Two 3 mm. fragments were cut from the larger stem and one each from the smaller stems. Under the binocular microscope, the distal end of the red piece was inserted into the proximal end of one of the large yellow pieces, and similarly the distal end of the small yellow fragment was inserted into the proximal end of the other large yellow piece. The original polarity was therefore maintained. Every effort was made to align the gastric ridges of the pieces, since preliminary experiments showed that failure to do so tended to prevent fusion of the fragments. It was considered preferable to insert the red stem into the yellow one rather than the converse, since it is possible that the hard perisarc of the insert might traumatize the coenosarc of the containing piece and liberate pigment. A few such converse experiments will be reported as controls. The use of the large yellow fragment as the containing piece also had the benefit of tending to make the hydranths form in the yellow component, since the fused 3 mm. fragments behave like a single 6 mm. stem piece in exhibiting dominance. The healing process is shown diagrammatically in Figure 1, A and B. One might largely insure formation of the hydranth in the yellow end by ligation of the red end, but ligation definitely damages tissue and so this was avoided. The colors of the regenerated hydranths were examined against a black background with strong white side lighting. The colors are unambiguous. The results are presented in Table I.

The significant results are those in which (1) successful fusion has occurred, with the coelenteron being common to both components, and (2) the formation of the hydranth has occurred in the yellow end of a red stem fused to a yellow stem. It will be noted that in every such case the hydranth was redder than the hydranth of the control consisting of two fused yellow pieces.

Observations of fused stems during the regeneration process show that the red pigment appears in the circulation following healing of the cut ends. It then seemingly is picked up by the gastric ridges. The gastric ridges of the yellow components can be seen with lines of red particles. This effect varies in uniformity since the gastric ridges do not occur symmetrically and the circulation currents vary in rate of flow and may have eddies. As noted by Stevens (1901) there is a tendency for the gastric ridges to break down as regeneration proceeds and the picked-up pigment tends to be re-liberated. When the primordia of the tentacles form, however, it can be inferred that pigment particles are picked up by them, as chains of these particles mark the tentacular ridges. There are also some randomly distributed particles in this area.

The above experiment was repeated in a few cases with oil drops placed in the coelenteron of the yellow components before healing occurred. These drops block

the circulation. In these cases the hydranth color was the same as that of the control hydranth. This might also be held to rule out a factor transmitted by the red component to the yellow via the coenosarc. The oil drop, however, may affect the coenosarc adjacent to it by limiting the entering and leaving of metabolically important materials, and thus effectively also block the coenosarc pathway.

TABLE I

A: Success of Fusions

All fusions successful and normal except:

Set No.	Yellow to Yellow	Yellow to Red
7	S	U
11	U, cytolyzed	U, abnormal
12	U	S
15	S, abnormal	S
26	U	S, abnormal

B: Observed Color of Hydranths

Fusion: Hydranth-bearing end: Set No.	Yellow in Yellow Either	Red in Red	Yellow in Yellow	Yellow in Red	Red in Red
1	Y	O	O		
2	Y	R			
3	O		OR		
4	O				OR
5	Y		O		
6	Y			Y	
7	O	R	Y		
8	YO	OR			
9	Y		O		
10	YO			OR	
11	—	O			
12	Y			OR	OR
13	Y	R			
14	YO		OR		
15	Y		O		
16	Y		O		
17	Y		OR		
18	Y		OR		
19	Y		O		
20	Y	OR			
21	Y		O		
22	YO		OR		
23	Y	OR			
24	Y	O	O		
25	Y	OR	O		
26	Y	OR			

KEY: S—successful; U—unsuccessful; Y—yellow; YO—yellow-orange; O—orange; OR—orange-red; R—Red.

Since one could not see individual particles being picked up, the possibility also remained that some soluble pigment precursor was being produced by the red stem and entering the yellow. Attempts to insert filters of various sorts into the coelenteron failed and the procedure was intrinsically undesirable, as it was impossible to place these without severe damage to the gastric ridges and partitions.

A probable resolution of these questions was effected in the following manner. "Norite" animal charcoal was ground in sea water with a mortar and pestle and a suspension of fine particles injected with a micropipette into the coelenteron of various stem pieces. These particles tend to adhere to the gastric ridges. Following healing, the particles become loose with breakdown of the ridge and are readily seen in circulation. This might simply be a washing free as circulation begins, but the disappearance of spot stains of Nile blue sulphate suggested an actual breakdown. With regeneration the charcoal particles tend to adhere in the area of hydranth formation and particle chains delineate the primordia of the tentacles. In one case where a stolon started to form, a tendency was noted for the charcoal particles to adhere at this area. In every respect the distribution of red pigment particles and charcoal particles is the same.

Smears of emerged hydranths following charcoal treatment show that the pigment masses and charcoal are similarly localized in the endoderm and between the endoderm and ectoderm layers. Sections of forming and emerged hydranths, following Zenker's fixation and staining with carefully filtered Heidenhain's haematoxylin without counterstain, confirm the charcoal distribution as observed in smears. Small charcoal fragments appear to be inside some endoderm cells. No mitoses were evident in the regenerating areas.

The above observations are regarded as making it highly probable that pigment particles are actually picked up. It was then decided to examine the character of the circulating fluid.

Stevens (1901) reports the contents of the circulation as pigment granules, translucent globules and nuclei. Hargitt (1903) records the presence in the same species of cnidocysts in the endoderm and circulation. We have examined the character of the fluid withdrawn from circulation with a fine pipette and confirm the presence of cnidocysts, pigment granules and cell debris. The material in circulation varies according to the time after healing when the fluid is examined. This may account for the failure of Stevens to find cnidocysts.

To determine the origin of the circulating material, smears of stem tissue were examined for obviously specialized cell types. In the endoderm were seen numerous cnidocyst cells and round or oval pigment cells. The latter are generally yellow but frequently have in them darker orange or red masses suggesting storage or concentration of this material.

In the course of regeneration, the area at an end where a hydranth is not forming becomes transparent; it was therefore decided to check to see whether this was an actual thinning or simply a loss of opaque material such as pigment. An actual thinning might be due to local cytolysis or a contraction of tissue into other areas, or to cell migrations. Therefore, sections of stems were cut and immediately thereafter additional cylinders approximately 1 mm. in length were cut from the remaining stem just proximal and just distal to the 5 mm. fragment. Working rapidly, these were immediately stood on end and the tissue thickness measured, using an ocular micrometer. The magnification was $43\times$ and 1 mm. of tissue was 10 micrometer units. This procedure is not adequate for small changes; however, it was readily observed that changes of the magnitude of a 50% decrease in coenosarc thickness occurred when, during regeneration, 1 mm. cylinders were cut from the stem piece up to the area of hydranth formation. It was not possible to judge with

certainly whether a gradient of thickness, showing increase in the direction of the hydranth, occurred although this is the visual impression. The need for speed in measurement is due to the fact that with the onset of the healing process the cut ends curl inward and increase in optical cross-section. The hydranth-forming area, as expected, becomes very thick and almost obliterates the coelenteron. Measurements were made on perisarc thickness concurrently as a check on technique, and these showed no change.

Chemical studies of a preliminary nature were then made on red stems, only, in an effort to obtain some information about the pigment. The pigment proved to be water insoluble but soluble in organic solvents, particularly polar solvents such as acetone, methanol and pyridine. With concentrated sulfuric acid a blue color is obtained. A chloroform solution gives a positive Carr-Price test for carotenoid by giving a blue-green color. Absorption measurements were made on various extracts using a Beckman spectrophotometer. In every case, single peaks in the visible range were obtained. These were: 490–500 $m\mu$ in pyridine; 455–70 $m\mu$ in petrol ether; and 470–80 $m\mu$ in acetone. The only carotenoid listed in Karrer and Jucker (1950) showing such characteristics is astacene. Astacene's solubility properties are very similar to those of the red pigment. Astacene is a pigment occurring mainly in animals, only one case being known of its presence in a plant, an alga. It is, however, supposedly an extraction artefact of astaxanthin and results from oxidation during extraction. Attempts to obtain astaxanthin by homogenizing hydranths in acetone-solid CO_2 slush and extracting under nitrogen failed but are not regarded as being final.

The above extracts were made on hydranths only, since numerous red algae and other organisms grow on the perisarc. It was noted, however, during the course of tissue smears, that our material was frequently infected with red algae in the endoderm. The most common of these resembles *Ceramium*.

As a preliminary to future work, an extract of hydranths of all colors in petrol ether was chromatogrammed on a sucrose column. Three red bands, one yellow band and one orange band were noted, but no attempt was made to collect the fractions. The bands may be due to different chemical chromatophores, to different esterified combinations of the same chromatophore, or to natural or extraction-created isomers.

DISCUSSION

Loeb (1891), who first noted the color changes in regeneration, suggested that the pigment might be nutritive. Driesch (1900), noting that the pigment particles seemed to be linearly arranged following the tentacular primordia, believed that they might have morphogenetic significance. Morgan (1901) challenged this, however, on the basis that despite evident considerable variation in pigmentation, regeneration proceeded normally. He also noted that the stolon frequently showed the pigment.

There is still no evidence of any functional significance for the pigment of *Tubularia*. The non-random arrangement of pigment particles seen by Driesch as delineating the tentacular primordia is now seen to be due to a mechanism which indiscriminately takes up particles. The presence of cnidocysts in the endoderm and in circulation suggests that this may be the normal method of supplying the

cnidocyst units of the new hydranth. The presence of charcoal particles between the two layers but not in the ectoderm would then suggest that the mechanism is particle-indiscriminate up to this point, but that incorporation into the ectoderm involves discrimination.

Against this concept it must be noted that charcoal and pigment tend to adhere where the stolon begins to form. This may mean that these growing areas simply have sticky surfaces and that the lining-up of particles in the tentacular primordia is the incidental result of cell movements.

Stevens (1901) and Godlewski (1904) believe that the red pigment is liberated by endoderm cytolysis, particularly of the gastric ridges, during regeneration, and is a waste material expelled when regeneration is complete. We confirm the liberation of some pigment with gastric ridge breakdown.

The pigment may indeed be a waste product derived from ingested crustaceans who, in all probability, contain astacene (or astaxanthin). There is no denial that some is frequently expelled after reconstitution. Preliminary chromatography does suggest, however, that it may pass through several chemical stages in *Tubularia*, as is also suggested by the occurrence of strikingly different colored specimens in the same clump of stems. These could be biochemical mutants.

The storage of pigment in *Tubularia* might serve as a prey-attracting mechanism. No evidence exists on this point. Carotenoid pigments are of known importance in visual biochemistry. There is no evidence on light sensitivity in this form. Carotenoid materials have been reported as gamete-attracting agents (Moewus, 1940). While this has not been confirmed, the pigment here occurs in both sexes and in apparently similar distribution.

Regarding the chemical nature of the pigment, Driesch (1900) remarks that it was identified for him as a carotenoid, and Lönnberg and Hellström (1931), working with *T. larynx*, name it as astacene. However, they report a main absorption band in petrol ether at 432-4 $m\mu$ and minor bands at 458-9 $m\mu$ and 488-91 $m\mu$. Our work confirms their identification but not the presence of three bands. Our data are in agreement with the spectral and other characteristics described by Karrer and Jucker (1950) for astacene.

It is undisputed that the area of hydranth formation shows a prior reddening, and the latter must be due to one or more of the following events: 1) local synthesis of pigment; 2) local acquisition of pigment formed elsewhere; and 3) more pigmented cells entering the area. Therefore, histological studies of the cell movements, divisions and growth relative to regeneration are important. Histological studies have been made by Bickford (1894), Stevens (1901), Godlewski (1904) and Hargitt (1903). Alone among these, Stevens reports mitotic figures in the regenerating area, both in ectoderm and endoderm. She also noted that in her sections the tissue in the non-regenerating area had become thin and the cells elongated in the stem length. To her, this suggested contraction of cells into the regenerating area. Contraction would account for reddening by increasing the length of the optical path through the pigmented material. This is readily duplicated by ramming tissue in the perisarc with a glass rod. Hargitt, unlike Stevens, finds no mitoses and believes that amitotic divisions must occur. Cell divisions are necessary to account for the increase in ectoderm surface, according to Hargitt. Bickford's work is directed towards the successful establishment of the view that the

hydranth must form from previously differentiated cells, rather than from a primitive cell, but she does not touch the problem of which differentiated cells form the hydranth. Against the contraction hypothesis of Stevens is the old observation of Allmann (1874) that no muscle cells exist in the tissue under the perisarc. This is not necessarily a valid objection since all cells are contractile to some degree.

Our work confirms the decrease in tissue thickness and the absence of distinguishable mitotic figures which therefore would lend weight to a cell movement theory. It must be pointed out, however, with regard to the observation regarding mitoses, that it is possible that we are dealing with mitoses of an unusual type. Mitoses might occur in waves, for example, and thus not be picked up in sections made on 5 or 6 stems. This would explain Stevens' finding them and the failure of the other observers to do so. Work with agents which arrest mitoses might clarify this point.

One still may not say that the reddening which marks the site of hydranth formation is entirely due to picked-up pigment. While this and intrinsic pigment could account for a good part of the reddening, contraction of tissue into the area is not ruled out. The discreteness of the particles marking the tentacular primordia, however, suggests that the former originate entirely from picked-up pigment.

SUMMARY

1. At least part of the reddening designating the site of hydranth formation in *Tubularia* is due to pigment removed from circulation.

2. The mechanism removing pigment particles from circulation does not distinguish them from charcoal particles introduced into the stem. These become distributed in exact coincidence with the pigment in the reconstituted hydranth.

3. The extracted pigment of *Tubularia* seems to be mainly astacene, as judged from its spectral and solubility properties.

4. The immediate origin of the pigment in circulation is from the breakdown of endodermal tissue.

5. The possible significance of removal of pigment is discussed.

6. Color variants in *Tubularia* are noted, as well as infecting red algae.

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